Journal of Medical Virology 61:70–75 (2000)

High Prevalence of Human Papillomavirus Type 16 Infection Among Children

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Infection with high-risk human papillomaviruses (HPV), is the most significant risk factor for cervical cancer and it may be possible to prevent this malignancy by immunisation. Before immunisation programmes can be designed, however, it is necessary to know the age of acquisition and all routes of infection for these viruses. Sexual transmission is well documented and vertical transmission has also been demonstrated, although the frequency of transmission remains controversial. We previously showed that vertical transmission frequently results in persistent infection, and now present data on the prevalence of HPV-16 DNA (the most prevalent high-risk HPV type) in healthy children. Buccal samples from 267 healthy children aged 3–11 years were tested for HPV DNA by generic PCR (MY09/MY11), and a HPV-16 specific nested PCR. Reverse transcriptase (RT)-PCR was used to determine the prevalence of transcriptionally active HPV-16 infection in a subset of children. HPV-16 DNA was detected by nested PCR in 138 of 267 (51.7%) samples, whereas HPV DNA was detected in only 45 (16.8%) specimens by generic PCR, that has a lower analytical sensitivity. There were no significant differences in prevalence according to age or sex. Early region mRNA was detected by RT-PCR in six (11.3%) of 53 HPV-16 E5 DNA positive samples. HPV-16 E5 DNA sequences from 10 children confirmed the identity of the sequences detected and identified 13 HPV-16 variants. ***J. Med. Virol. 61:70–75, 2000.***

KEY WORDS: HPV-16; PCR; prevalence; analytical sensitivity

INTRODUCTION

There are more than 100 human papillomavirus (HPV) types, 30 of which infect mucosal epithelia [van Ranst et al., 1996]. These are divided into high-risk types (e.g., HPV-16 and -18) associated with anogenital carcinomas and low-risk types (e.g., HPV-6 and -11) that cause benign genital warts. Infection with high-risk HPVs is the most significant risk factor for cervical cancer [Koutsky et al., 1992; Schiffman et al., 1993], with HPV DNA detectable in at least 95% of cases [Bosch et al., 1995]. It may soon be possible to prevent cervical cancer by prophylactic immunisation against high-risk HPVs [Braly 1996; Galloway 1996]. It is imperative, however, to determine the age of acquisition and all routes of infection with high-risk HPVs in order to decide at what age to offer immunisation.

Cervical cancer and cervical intraepithelial neoplasia (CIN) have the epidemiology of a sexually transmitted disease. Although it is established that high risk HPVs are transmitted sexually [Ley et al., 1991; Wheeler et al., 1993], there is evidence that non-sexual transmission also occurs [Cason et al., 1996]. Vertical transmission of high risk HPVs, that was associated with a high viral load in the genital tract during pregnancy has been demonstrated [Kaye et al., 1994; Cason et al., 1995]. Furthermore, we confirmed by DNA sequencing that the mother was the source of infection and that infection persisted for at least 2 years after birth [Kaye et al., 1996] with evidence of virus transcription in some children [Cason et al., 1999]. Vertical transmission has been confirmed by others [Fredericks et al., 1993; Puranen et al., 1997; Tseng et al., 1998].

Despite these observations, some workers are reluctant to accept the occurrence of vertical transmission and infection of pre-pubertal children with high-risk HPVs [Koch et al., 1997; Watts et al., 1998; Dillner et al., 1999]. Therefore, we have tested 267 healthy children using both generic and type-specific polymerase chain reaction (PCR) assays of differing sensitivity, to allow comparison with previous studies [Koch et al.,

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Accepted 4 October 1999

TABLE 1. Age and Sex Distribution of Children

Age (years)	3	4	5	6	7	8	9	10	11	Total
Boys	9	16	24	13	16	16	14	20	8	136
Girls	10	14	7	17	14	17	23	23	6	131
Total	19	30	31	30	30	33	37	43	14	267

1997; Mund et al., 1997; Puranen et al., 1997]. The frequency of transcriptionally active HPV-16 infection was also assessed.

MATERIALS AND METHODS

Study Population and Samples

Two hundred and sixty-seven children (136 male), aged 3–11 years were recruited from three state primary schools and a day nursery in the inner London area served by our hospital (Table I). These included 119 siblings from 54 families. Parents were sent a letter of consent and questionnaire on which to record the child's details. Approval for the study was obtained from the Research Ethics Committee of St. Thomas' Hospital.

Buccal samples were collected from groups of 5–8 children under supervision. Children brushed their buccal mucosa on both sides for 30–45 seconds with a cytobrush (Axibrush, TM Colgate medical) after an investigator (PR) had demonstrated the technique. Buccal cells from swabs were dispersed into bijoux bottles containing 5 ml of sterile distilled water (molecular biology grade). For each collection group, one bottle was inoculated with an unused cytobrush to act as a control; a total of 40 such controls were collected. Each sample was divided into five 1 ml aliquots and stored at −70°C. To determine if HPV DNA could be detected in the school environment, 20 swabs were taken from two schools (floor [3], tables [3], door handles [2], toys [2], sinks [3], taps [3], tap water [2] and toilet seats [2]).

Buccal cells from each 1 ml aliquot were pelleted by centrifugation, resuspended in 200 μl of Proteinase K digestion buffer containing 0.45% (v/v) NP-40TM (Sigma, UK), 0.45% (v/v) Tween-20TM (Sigma) and 60 μg/ml Proteinase K (PK; Boehringer Ltd, UK) and incubated overnight at 55°C. PK was subsequently inactivated by heating at 90°C for 10 minutes. Immediately prior to PCR, each sample was vortexed for 10 seconds to ensure an even distribution of material. A 5 μl aliquot was used in all PCR reactions (total volume 50 μl).

Polymerase Chain Reactions

MY09/MY11. This generic PCR is capable of detecting most cutaneous and mucosal HPV types [Manos et al., 1989]. It amplifies a portion of the L1 ORF using degenerate primers to give an amplicon of ≈450 bp. In our hands it has an analytical sensitivity of between 25 and 250 copies of HPV-16 DNA [Cavuslu et al., 1996a].

HPV-16 nested PCR. HPV-16 DNA was detected using a type-specific nested PCR that amplifies a region in the E5 open reading frame (ORF) [Cavuslu et al., 1996b]. This PCR detects <10 copies of HPV-16 DNA [Cavuslu et al., 1996a]. Five μl of the PK digest was used in the first round of PCR and 2 μl of the first round product was used in the nested reaction.

β-globin PCR. To confirm that the samples were of sufficient quantity and quality for PCR and to detect PCR inhibitors, all samples were tested for β-globin DNA by PCR using primers GH20 and PC04 [Saiki et al., 1986]. After amplification, 18 μl of PCR product was electrophoresed and visualised on 2% (w/v) agarose gels containing 0.5 g/l ethidium bromide. All methods have been described in detail elsewhere [Mant et al., 1997].

RT-PCR. To determine whether detection of HPV-16 DNA was representative of transcriptionally active infection, 76 samples chosen at random, were tested for early region mRNA transcripts (E-mRNA) by RT-PCR [Biswas et al., 1997].

PCR precautions. In all PCRs stringent precautions were taken to avoid contamination. Sample collection and PCRs were not performed in the same week; four geographically remote laboratories were used for reagent preparation, sample preparation, amplification and gel analyses [Mant et al., 1997].

PCR controls. Negative controls included a PK digest without template DNA and water (molecular biology grade) blanks and a control swab for each 8 samples tested. An HPV DNA negative ovarian carcinoma cell-line (A431) was used as a negative human cell control, whereas the positive control was an HPV-16 containing cell line (SiHa cells) and/or plasmid pAT16 [Mant et al., 1997]. If the negative cell control, PK or water blanks gave positive results, the entire run was discarded.

DNA sequencing. DNA from samples positive in the first round of E5 PCR (nucleotides 3837–4109) were ligated into pGEM-T (Promega Ltd) and transformed into E.coli JM109 cells. Recombinant clones were sequenced in both orientations using SP6 and T7 primers flanking the insert, using an ALF DNA sequencer (Pharmacia). DNA amplified from the pAT-16 reference HPV-16 isolate and cloned from CaSki, a HPV-16 DNA positive cervical cancer cell line, were also sequenced.

Statistical analysis. Prevalence of HPV DNA was analysed by age and sex using Fisher's exact test. McNemar's test and the kappa test were used to compare the detection of HPV DNA by the two assays.

RESULTS

Prevalence of HPV-16 DNA Including Concordant Infection Among Siblings and Results From Environmental Samples

All buccal samples were positive for β-globin DNA. HPV DNA was detected in 157 of the 267 (58.8%) buccal samples by at least one PCR (Table II). HPV-16 DNA was detected in 138 (51.7%) samples by type-specific PCR, whereas HPV DNA was detected in only 45 (16.8%) samples by generic PCR with MY09/MY11 primers (Tables II & III). There were no significant differences in prevalence of HPV infection according to

TABLE II. Detection of HPV DNA in Buccal Samples Using Generic and Type-Specific PCRs

PCR method	Analytical sensitivity of PCR method	Number (%) HPV DNA positive		
		Boys	Girls	Total
MY09/MY11	25–250	19/136 (14.0%)	26/131 (19.8%)	45/267 (16.8%)
HPV-16 E5 (Nested)	2.5	76/136 (55.9%)	62/131 (47.3%)	138[a]/267 (51.7%)
Positive by any assay		84/136 (61.8%)	73/131 (55.7%)	157/267 (58.8%)

Results of McNemar's (with Yate's correction) test for comparison of the HPV detection rate by individual PCRs: E5 nested v MY09/MY11; $P < 0.0001$. Genome copies: Cavuslu et al. 1996a.
[a]Fifty-eight (42%) of these samples were positive in the first round of PCR.

TABLE III. Comparison of Results Obtained With Generic and Type-Specific PCRs

PCR result with primer set		Number of children with these results
Generic MY09/MY11	Type specific HPV-16 E5 nested	
–	–	110
+	–	19
–	+	112
+	+	26
Total		267

age (Fig. 1) or sex (Table II). One hundred and twelve of the 267 samples (42%) were positive only in the HPV-16 nested E5 PC, whereas 19 (7.1%) of the samples were positive only in the generic PCR (Table III). The differences in the detection rate of HPV DNA by the two assays were statistically significant (Table II).

HPV-16 DNA results by nested PCR were available from 54 families with 2 or more children attending school. HPV-16 DNA was detected in both siblings in 13 (29.5%) of the 44 families with 2 children, whereas both siblings in 11 (25%) families were HPV negative. Thus, concordant results were obtained in 24 of 44 (55%) sibling pairs. Concordant results were also obtained from 5 of 9 (56%) families with 3 children, and in the one family with 4 children, all were HPV-16 DNA positive. Among 6 pairs of twins, 3 (50%) were both HPV-16 DNA positive, 2 pairs were HPV-16 negative, and one pair gave discordant results.

HPV-16 DNA was not detected in any of the 20 environmental samples or 40 control swabs.

Evidence for HPV-16 Transcription

Of the 76 samples tested by RT-PCR, 53 were HPV-16 DNA positive and of these, six (11.3%) contained detectable E-mRNA. The 23 HPV-16 DNA negative samples were negative for E-mRNA. The age distribution and mean age of E-mRNA positive and negative children did not differ significantly.

DNA Sequencing

Nineteen cloned HPV DNA sequences (Genebank accession numbers AJ244840, AJ244857-62, AJ244864, AJ244868-71, AJ244874-80) obtained from 10 children were confirmed as HPV-16 E5 as all had >98% homology with the reference isolate of HPV-16 [Seedorf et al., 1988]. Thirteen different HPV-16 E5 sequences were demonstrated; HPV DNA sequences from pAT-16 and CaSki cells revealed no deviation from the published sequences when both were sequenced 10 times in both orientations. In six children with two or more clones, two had identical sequences and four had two or more different sequences, suggesting infection with more than one variant.

DISCUSSION

This study provides evidence for mucosal HPV infection in children. In many children low levels of viral DNA are present, which were detected only with a very sensitive PCR. Using nested PCR, HPV-16 DNA was detected in 51.7% buccal swabs from 267 3-11-year-old children, and in some cases infection was shown to be transcriptionally active. We are convinced that our results represent accurately HPV-16 infection in children for three reasons: 1) positive results were not due to laboratory or environmental contamination as stringent precautions were employed to prevent cross contamination in the laboratory (see methods), and no environmental or PK digest controls or PCR reagent controls, interspersed randomly with samples, tested positive; 2) sequencing of HPV DNA from buccal swabs revealed several variants of HPV-16 E5 in children, proving that the results were not due to laboratory contamination from a single source; and 3) transcriptionally active infection was demonstrated in at least 11% of HPV-16 DNA positive children, that may be an underestimate of active infection, as the sensitivity of the RT-PCR used to detect E-mRNA (2000 mRNA copies [Biswas et al., 1997]) is significantly less than the DNA PCR (<10 genome copies per reaction). It should be noted that high-risk HPV DNA has also been detected in the oral cavity of 22–44% adults [Jalal et al., 1992; Kellokoski et al., 1992; Puranen et al., 1996; Badaracco et al., 1998]. HPV-16 DNA has been detected in cervical brush-scrapes from 40% women attending well women clinics in our area of London [Biswas et al., 1997].

By using two PCRs, it was demonstrated that the rate of detection of HPV-16 DNA is dependent on the sensitivity of the assay used. There was poor agreement between the two assays (kappa statistic 0.039) as they differ in both sensitivity and specificity. HPV-16 DNA was detected in 112 (42%) of the samples only by HPV-16 nested PCR, demonstrating the sensitivity of that assay. Nineteen (7.1%) samples were positive only by the generic PCR, suggesting that types other than

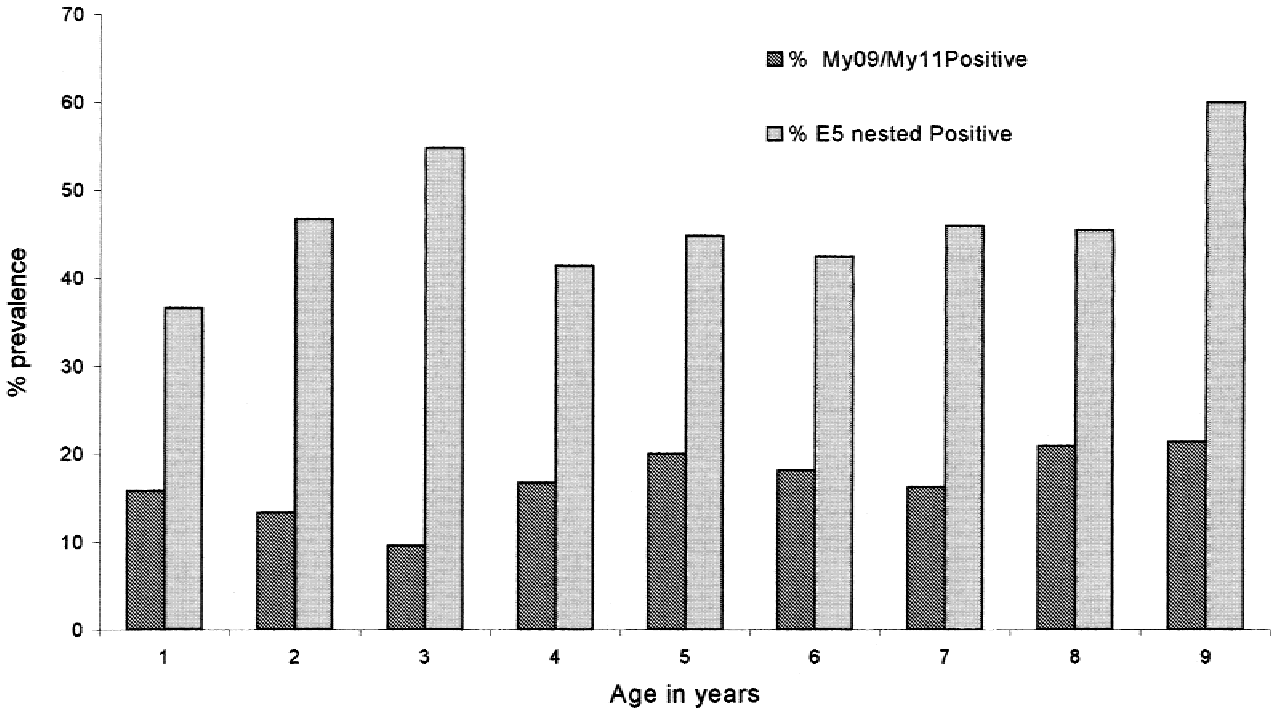

Fig. 1. Prevalence of HPV DNA in buccal swabs from children using MY09/MY11 and HPV-16 E5 nested CR; n = number of children per age group.

HPV-16 were present in these samples. Other studies, in which type-specific PCRs were used, have reported up to 26% prevalence of HPV-16 DNA in children [Jochmus-Kudielka et al., 1989; Jenison et al., 1990; Mund et al., 1997], whereas Puranen and colleagues [1996] detected HPV DNA in oral scrapings from 31.6% children using generic primers. Other groups, using generic PCRs, have failed to detect HPV DNA in samples from children [Koch et al., 1997; Watts et al., 1998] and virgins [Fairley et al., 1992]. Although these disparities may reflect population differences, this study indicates that differences in the sensitivity and specificity of the PCR assays employed are at least as important. Type-specific PCRs are 2–10-fold more sensitive than generic PCRs [Baay et al., 1996; Cavuslu et al., 1996a] and the use of only one generic primer pair may lead to an underestimation of the prevalence of infection [Karlson et al., 1996; Qu et al., 1997]. Results may also be influenced by the use of DNA extraction procedures, the volume of sample tested, the number of PCR cycles and other variations in PCR methods employed. Thus, there is a need for quality control mechanisms to allow meaningful comparison of results from different laboratories.

In this cross-sectional study there were no significant differences in HPV prevalence according to age among children aged 3 to 11 years, suggesting that infection becomes established in early life and persists thereafter. Alternatively repeated infections may occur, to sustain prevalence at a constant rate. It was shown previously, by DNA sequencing, that virus may persist for at least the first two years of life in children who acquired infection from their mothers [Kaye et al., 1996].

Our previous results also suggest that horizontal infection may occur in infancy as mucosal HPV DNA was detected in 1 of 11 (9%) of 6-month-old infants who were previously HPV DNA negative and born to HPV negative mothers [Cason et al., 1995]. Puranen et al. [1996] also identified HPV DNA in buccal brush-swabs from children aged 0.3–11 years, who were born to HPV negative mothers. Other sexually transmitted virus infections may also be acquired in infancy (e.g., herpes simplex, cytomegalovirus, hepatitis B virus, HIV). The JC polyomavirus, closely related to the papillomaviruses, is frequently transmitted from parents to children in whom persistent infection is established [Kunitake et al., 1995; Kitamura et al., 1997]. Concordant results were obtained for 54% siblings in this study and further work is in progress with families to determine if there is evidence of horizontal infection.

Serological studies support childhood infection as antibodies to HPV-16 virus-like particles have been detected by enzyme immunoassays (EIAs) in 4–14% of children aged 1–13 years [Luxton et al., 1997; Marais et al., 1997; Cubie et al., 1998]. Detection of IgM antibody to a capsid protein (L2) of HPV-16 among 50% of children aged 1–10 years is further evidence for recent or persistent infection [Cason et al., 1995]. Others have failed to detect antibodies in children [Af Geijersstam et al., 1999], but this may be due to differences in the EIA protocols employed, including the choice of positive/negative cut-off.

Identification of vertical transmission and childhood

infection with high risk HPVs has added to our understanding of the epidemiology of these infections. Although the consequences of vertical or early childhood infection are as yet unknown, vertical transmission of high risk HPVs may induce HPV specific humoral and cell-mediated immunity, or specific immune tolerance. If tolerance is acquired, prophylactic HPV vaccines may have to be offered at birth to prevent sexually-acquired infection later in life. Indeed, children exposed to hepatitis B virus at birth may acquire immune tolerance and failure of specific immune responses results in persistent infection in 70%–90% cases. Hepatitis B vaccine given at birth has been shown to prevent infection and has already reduced the incidence of childhood hepatocellular carcinoma in Taiwan [Chang et al., 1997]. Before HPV vaccine programmes can be introduced, further information is required on HPV specific immune responses in children and the importance of different routes of transmission among children and families. Such studies are already in progress in our laboratory.

ACKNOWLEDGMENTS

We are grateful to the Wellcome Trust and Smith's Charity for financial support and to the head teachers, staff, parents and children of the three schools and the nursery involved. We also thank Professor J.E. Banatvala (Department of Virology, GKT) for constructive discussion when preparing this manuscript.

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